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TRIM27/MRTF-B-Dependent Integrin β1 Expression Defines Leading Cells in Cancer Cell Collectives

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SUMMARY

For collective invasion, cancer cells form cohesive groups comprised of leading cells (LCs) at the forefront and following cells (FCs) at the rear. However, the molecular mechanisms that define LCs and FCs remain elusive. Here, we demonstrated that LCs, but not FCs, upregulated the expression of integrin β1 after the loss of intercellular adhesion. The LC-specific expression of integrin β1 was posttranscriptionally regulated by the TRIM27/MRTF-B complex in response to the loss of intercellular adhesion, thereby regulating the stability and translation of *integrin β1* mRNA via *microRNA-124* in LCs. Accordingly, depletion of TRIM27 and MRTF-B abrogated the upregulation of integrin β1 in LCs and blocked the invasion of cancer cell groups in vitro and in vivo. Therefore, our findings revealed that the specific function of LCs was defined by intrinsic mechanisms related to the presence of the cell's free surface, providing insights into the regulation of intratumor heterogeneity.

INTRODUCTION

Metastasis of a primary tumor to different tissues and organs is generally the cause of cancer-related death. During cancer progression, invasion of cancer cells is the first step required for metastasis (Thiery, 2002; Sahai, 2007). Numerous studies have postulated that invasion into the surrounding stroma requires cancer cells with epithelial cell morphology to undergo a phenotypic conversion termed the epithelial-mesenchymal transition (EMT), wherein they lose their intercellular adhesion ability and acquire mesenchymal morphology and increased invasion potential (Thiery, 2002; Hanahan and Weinberg, 2011; Scheel and Weinberg, 2012). Although this hypothesis is widely accepted, most pathologists have not observed cells in transition in human cancer tissues, leading some to suggest that the EMT program is activated in cancer cells instantaneously over short periods of time or is observed only in limited contexts (Friedl and Wolf, 2003; Tarin et al., 2005; Friedl and Alexander, 2011). Instead, rigorous pathological studies have long suggested that cancers form tightly connected groups of cells in order to invade into neighboring tissues, a phenomenon termed collective cell invasion or migration (Yamamoto et al., 1983; DiCostanzo et al., 1990; Sahai, 2005; Friedl and Gilmour, 2009; Gray et al., 2010; Friedl et al., 2012; Rørth, 2012). The observation that grouped cancer cells circulate in the blood stream of cancer patients further suggests that groups of cancer cells may penetrate into the walls of blood or lymphatic vessels (Hart, 2009; Hou et al., 2011). In line with these observations, an experimental animal model showed that cancer cells undergoing collective invasion were prone to lymphatic dissemination (Giampieri et al., 2009). Notably, collective migration is not limited to cancer cell invasion and metastasis; it is also a major feature of embryonic morphogenetic events and tissue repair, thus indicating that there are likely to be common mechanisms mediating these cell migratory processes (Rørth, 2012; Mayor and Carmona-Fontaine, 2010; Theveneau and Mayor, 2012).

Although collective cell invasion and migration have attracted much attention in recent years, the underlying mechanisms, which are distinct from single-cell invasion/migration, have not been fully resolved. Differences may lie in the heterogeneity among individual cells that constitute the cell groups (Rørth, 2012; Easty and Easty, 1974; Kolega 1981). Recent studies have shown that cancer cell groups consist of leading cells (LCs), which are located at the forefront of the group, and following cells (FCs), both of which invade en masse into the surrounding stroma (Friedl and Gilmour, 2009; Gray et al., 2010; Rørth, 2012). A recent study of the collective migration of nascent neural crest cells showed that LCs and FCs exhibit distinct gene expression patterns in response to external stimuli

and/or intercellular contact in order to generate locomotive activity and directionality (McLennan et al., 2012). Because cancer cells emulate the traits of collective migration that occurs as part of normal morphogenic events during development, it is likely that the differential gene expression profiles between LCs and FCs in cancer cell groups would also regulate their invasive potential (Rørth, 2012). Indeed, several studies have shown that the expression of some promigratory proteins, such as podoplanin and fascin, and the activity of the Rho family of small guanosine triphosphatases (GTPases) are differentially regulated in LCs (Wicki et al., 2006; Vignjevic et al., 2007; Carmona-Fontaine et al., 2008; Theveneau et al., 2010; Hidalgo-Carcedo et al., 2011). However, at present, it remains unclear whether the differences between LCs and FCs are determined by extrinsic signals, such as growth factors and cytokines derived from the stroma, or by the segregation of intrinsic factors during the invasion of cancer cell groups.

In the present study, we address the mechanism through which LCs, but not FCs surrounded by other cells, detect the presence of free surface in contact with the space around them and translate this into an LC-specific protein expression pattern. Our findings showed that the position of cells within cancer cell groups and their intrinsic machinery were both integrated to determine the gene expression of individual cells, thereby explaining the significance of cancer cell heterogeneity during cancer progression.

RESULTS

Tripartite Motif-Containing 27 Was Required for Upregulation of Integrin β1 in LCs

Integrin β1 is preferentially expressed in LCs, as identified by previous studies of in vitro 3D cell culture models (Hegerfeldt et al., 2002; Friedl et al., 2004) and histopathological observations of cancer tissues (Brockbank et al., 2005; An et al., 2013). Here, we immunostained human skin squamous cell carcinoma (SCC) tissues using anti-integrin β1 antibodies, revealing dominant expression of integrin β1 in LCs, but not in FCs, in many cases (8/13; 61.5%; Figures 1A and S1A). Interestingly, LC-specific integrin β1 expression was recapitulated in migrating groups of human SCC A431 cells cultured in a monolayer, as shown by immunofluorescence (Figure 1B). In these migrating cells, integrin β1 was localized in the focal adhesions in LCs (Figure 1B, right panel). Western blot analysis using lysates obtained from LCs and FCs that were differentially isolated from methanol-fixed cells using laser capture microdissection (LCM) also supported the observation of upregulated integrin β1 in LCs (Figures 1C and 1D).

We next investigated the mechanisms through which LCs specifically upregulate integrin β1. We previously showed that the transcriptional repressor tripartite motif-containing 27 (TRIM27) (also known as RET finger protein) is highly expressed in many types of cancers, but not in normal tissues, and that TRIM27 expression levels are highly correlated with poor prognoses in patients with colon, endometrial, and lung cancers (Takahashi et al., 1988; Shimono et al., 2000; Kato et al., 2009; Tsukamoto et al., 2009; Iwakoshi et al., 2012). Moreover, TRIM27 depletion by RNAi results in downregulation of integrin β1 in cancer cells (Tsukamoto et al., 2009), suggesting that TRIM27 may be involved in the differential expression of integrin β1 between LCs and FCs. To test this, control and TRIM27-depleted A431 cells were seeded at a low density to promote the formation of nests or clusters and were then allowed to grow to approximately 30% density to prevent the effects of cell confluence (Figure 1E) and to create more cells with a free surface in contact with the space around them. After normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, integrin β1 expression was significantly higher in A431 cells seeded to form nests compared with that observed in confluent cells. Additionally, this effect was abrogated by TRIM27 depletion (Figure 1F). Under the same conditions, integrin β1 expression in LCs, but not in FCs, which were isolated by LCM, was also reduced by TRIM27 depletion (Figures 1G and S1B). Interestingly, integrin β1 expression was also upregulated in a time-dependent manner by extensively scratching the confluent monolayer of A431 cells to create more cells with a free surface (Figures 1E and S1C), which we showed was also dependent on TRIM27 (Figure 1H). Thus, TRIM27 specifically regulated integrin β1 expression in LCs or cells with a free surface, but not in FCs or cells surrounded by other cells, in a monolayer cell culture model. Moreover, integrin α5, but not α2, αV, or β4, was upregulated in cells seeded to form nests, implying that certain combinations of integrins and their ligand-binding specificities may be important for the function of LCs (Figure S1D).

Interaction between TRIM27 and MRTF-B Regulated Integrin β1 Upregulation in LCs and Collective Migration of Cancer Cell Groups

Next, we hypothesized that changes in actin dynamics, which follow the generation of a cell's free surface (Hall, 2009), induce integrin β1 expression in LCs via TRIM27. Recently, the myocardin-related transcription factor (MRTF) family of proteins, which act as serum response factor (SRF) cofactors, were shown to regulate gene expression in response to changes in the polymerization equilibrium of monomeric globular (G) actin and filamentous (F) actin (Miralles et al., 2003; Selvaraj and Prywes, 2003; Posern and Treisman, 2006; Olson and Nordheim, 2010). Therefore, MRTF proteins may mediate integrin β1 expression in LCs. Indeed, the upregulation of integrin β1 induced in cells seeded to form nests (30% density) was attenuated by MRTF-B depletion in A431 cells (Figure 2A). The significance of MRTF-B in defining the function of LCs was also shown in experiments using LCs and FCs isolated by LCM; integrin β1 expression in LCs, but not FCs, was attenuated by MRTF-B depletion (Figures 2B and S2A). Immunostaining showed that approximately 30% of MRTF-B tagged with G196 (Kamata et al., 2013) localized in the nucleus in LCs, whereas MRTF-B was mostly in the cytoplasm in FCs of collectively migrating A431 cells (Figure 2C), consistent with the previous finding that MRTF proteins shuttle between the nucleus and cytoplasm in response to various stimuli (Posern and Treisman, 2006; Olson and Nordheim, 2010; Busche et al., 2008; Charbonney et al., 2011).

MRTF-B and TRIM27 colocalized in the nuclei of LCs (Figure 2D), and their physical interaction increased following

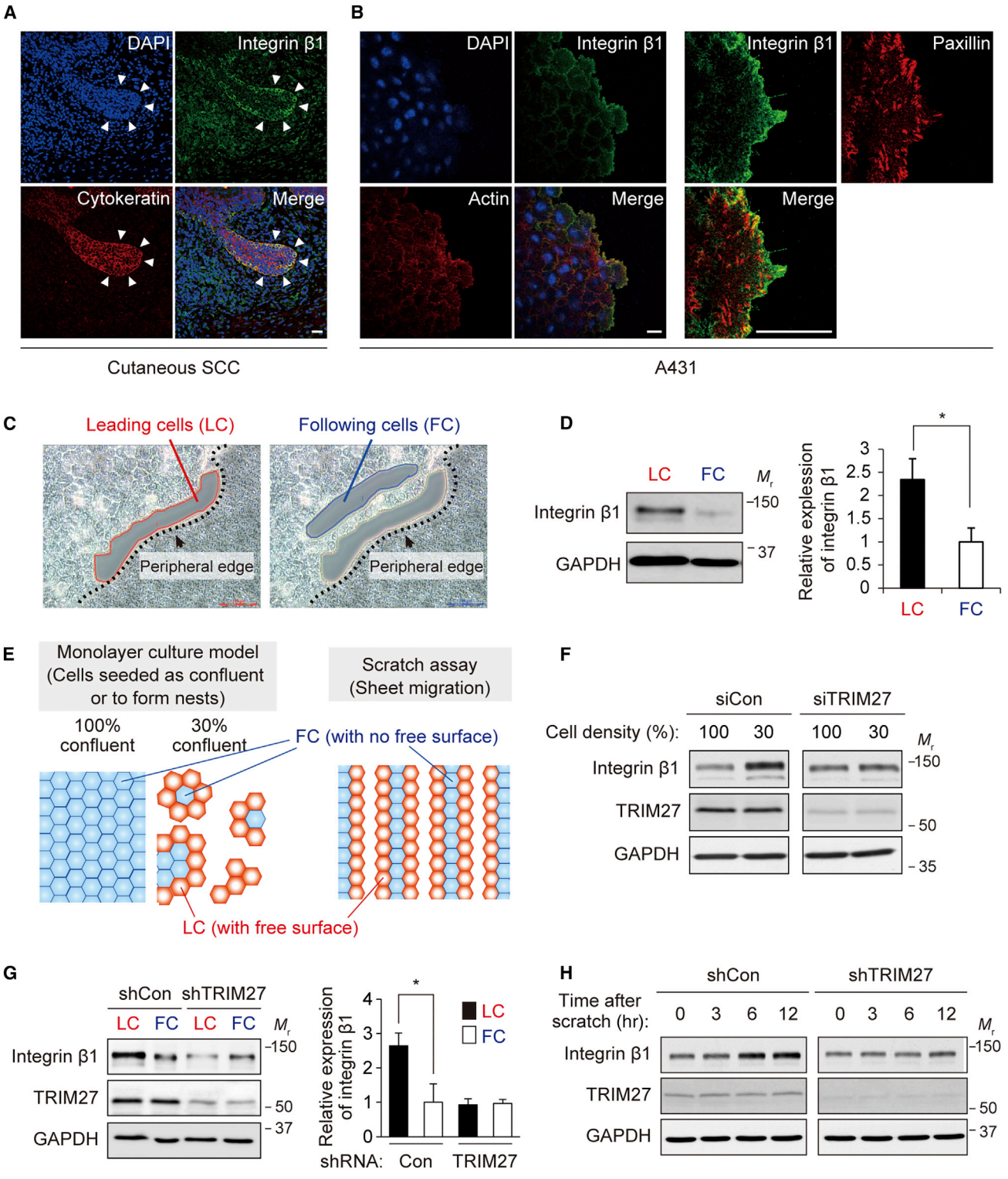

Figure 1. TRIM27 Was Required for Integrin β1 Upregulation in LCs

(A and B) Immunofluorescent staining with indicated antibodies in tissue sections obtained from a cutaneous SCC patient (A) and the SCC cell line A431 selected to undergo collective sheet migration (B). Arrowheads indicate the peripheral edge of a cancer nest. The right panel of (B) shows higher-magnification images. The scale bars represent 20 μm.

(C) Representative images of the isolation of LCs and FCs by LCM. Dashed lines indicate the peripheral edge of collectively moving cells.

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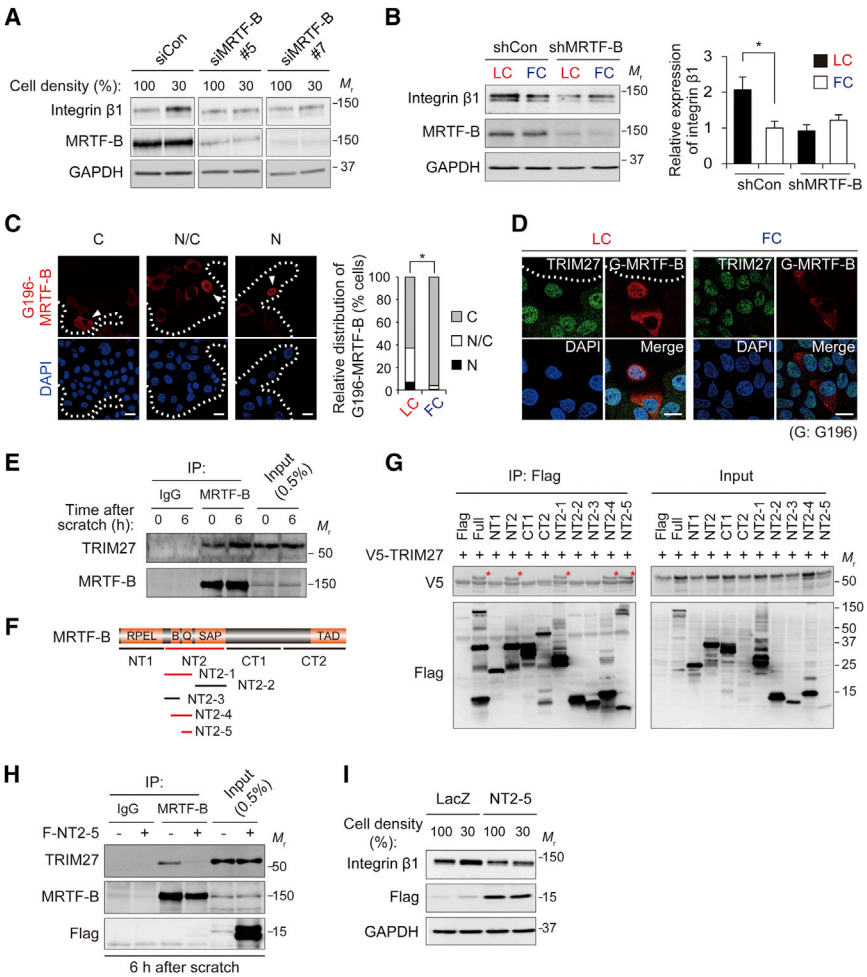

Figure 2. The Interaction between TRIM27 and MRTF-B Regulated Integrin β1 Upregulation in LCs and the Collective Migration of Cancer Cell Groups

(A and B) Effects of MRTF-B depletion in A431 cells seeded to form nests (A) and LCs isolated by LCM (B) on integrin β1 upregulation. The right panel of (B) shows quantification of western blot analyses (mean ± SD). *p < 0.05. shMRTF-B, small hairpin MRTF-B; siMRTF-B, small interfering MRTF-B.

(C) MRTF-B translocation to the nucleus in LCs. Representative images of the subcellular localization of G196-MRTF-B in LCs are shown. The right panel shows quantification of G196-MRTF-B localization in LCs and FCs. The scale bars represent 20 μm. For each group, 150 cells from three independent experiments were counted. *p < 0.05. C, cytoplasm; N, nucleus; N/C, nucleus and cytoplasm.

(D) TRIM27 colocalized with MRTF-B in the nucleus of LCs, but not FCs. The scale bars represent 20 μm.

(E) The interaction between TRIM27 with MRTF-B during sheet migration. Lysates obtained from A431 cells at the indicated time after scratching were immunoprecipitated with anti-MRTF-B followed by western blotting with the indicated antibodies.

(F) Schematic illustration of the domain structure of human MRTF-B. The red bars indicate TRIM27-interacting fragments.

(G) Interaction between TRIM27 and the NT2-5 region of MRTF-B. Lysates obtained from HEK293 cells transfected with the indicated plasmids were immunoprecipitated with FLAG antibodies.

(H and I) Effects of FLAG-NT2-5 overexpression on the interaction between TRIM27 and MRTF-B (H) and the upregulation of integrin β1 in LCs (I).

extensive scratching of the A431 cell monolayer, as shown using immunoprecipitation (IP) (Figure 2E). Domain mapping revealed that the glutamine-rich domain (Q domain; termed NT2-5; Posern and Treisman, 2006; Olson and Nordheim, 2010) of MRTF-B, but not the B domain responsible for SRF binding, contained a TRIM27-binding site, indicating that the interaction between MRTF-B and TRIM27 did not disrupt binding to SRF, which is essential for its transcriptional activity (Figures 2F and 2G; Miralles et al., 2003; Selvaraj and Prywes, 2003; Posern and Treisman, 2006; Olson and Nordheim, 2010). Overexpression of the NT2-5 domain, which competed with endogenous MRTF-B in binding to TRIM27 (Figure 2H), abrogated integrin β1 upregulation in cells seeded to form nests, but not confluent cells, in the monolayer cell culture model (Figures 2I and S2B). These data suggested that MRTF-B regulated integrin β1 expression through its interaction with TRIM27.

Loss of Intercellular Adhesion in LCs Induced TRIM27/MRTF-B-Mediated Integrin β1 Upregulation through Activation of Rho GTPase

In contrast to A431 cells, which retain tight intercellular adhesion, we found that integrin β1 expression was prone to regulation by TRIM27 in MDA-MB-231 breast cancer cells, which shows no E-cadherin expression with mesenchymal cell morphology (Graff et al., 1995), even in confluent cultures (Figure S3A), suggesting that this process may depend on E-cadherin-mediated intercellular adhesion. Accordingly, E-cadherin depletion and/or treatment with an E-cadherin neutralizing antibody (SHE78-7)

(D) Integrin β1 expression in isolated LCs and FCs. Quantification of data after normalization to GAPDH is shown in the right panel (mean ± SD). *p < 0.05.

(E) Schematic illustration of the experimental models. Left, A431 cells were seeded to form a confluent monolayer (100% confluent) or nests (30%). Right, the cell monolayer was scratched to induce sheet migration.

(F–H) Effects of TRIM27 depletion on integrin β1 expression in A431 cells seeded to be confluent or form nests (F), in LCs isolated by LCM (G), and in cells selected to undergo collective sheet migration induced by extensive scratching of the monolayer (H). The right panel of (G) shows quantification of western blot analyses (mean ± SD). *p < 0.05. shCon, small hairpin control; shRNA, small hairpin RNA; shTRIM27, small hairpin TRIM27; siCon, small interfering control; siTRIM27, small interfering TRIM27.

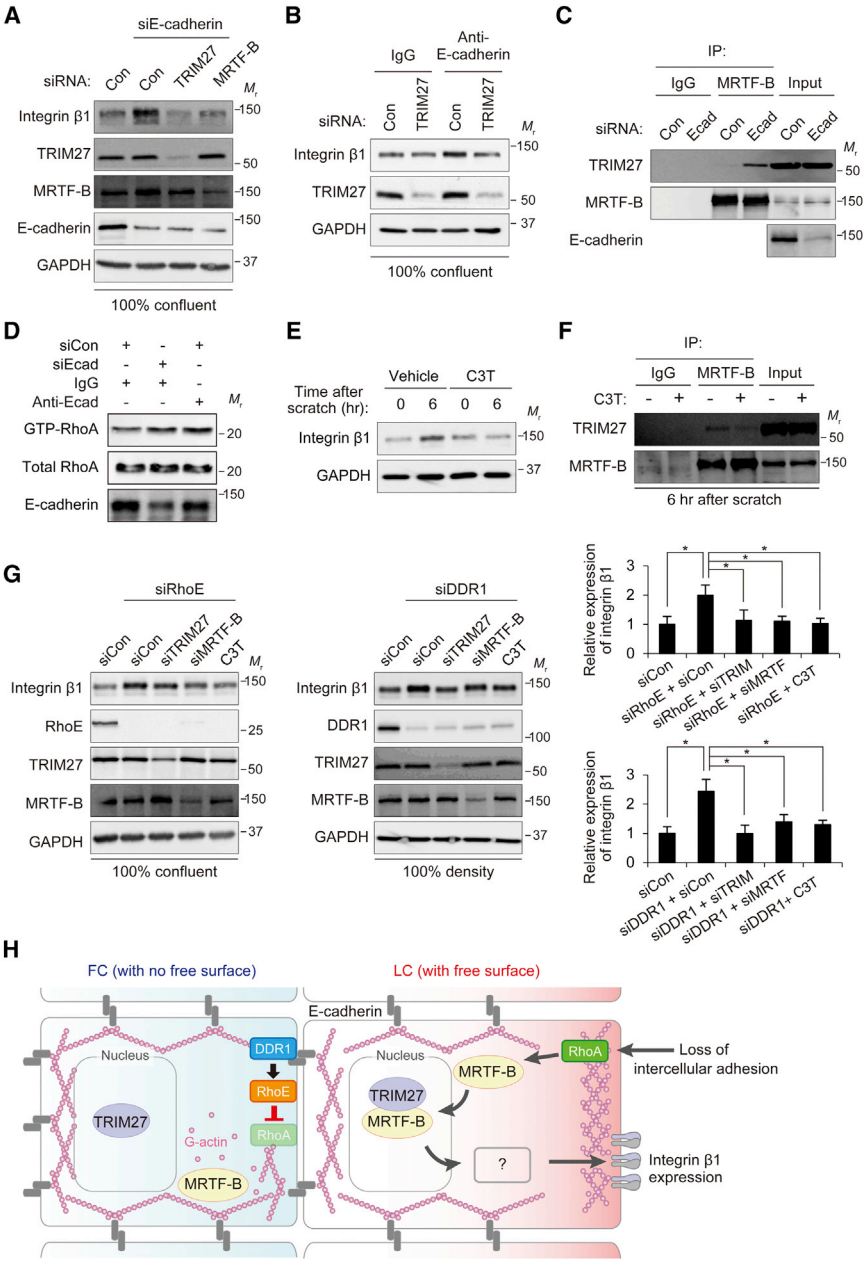

Figure 3. Loss of Intercellular Adhesion in LCs Induced TRIM27/MRTF-B-Mediated Integrin β1 Upregulation

(A and B) Effects of depletion (A) or inhibition (B) of E-cadherin on TRIM27/MRTF-B-dependent upregulation of integrin β1 in confluent monolayers of A431 cells. A431 cells transfected with E-cadherin small interfering RNA (siRNA) and either control, TRIM27, or MRTF-B siRNA (A) or those treated with either E-cadherin-neutralizing antibodies or control IgG (B) were seeded for confluence in a monolayer and subjected to western blot analysis. siE-cadherin, small interfering E-cadherin.

(C) Effects of E-cadherin depletion on the interaction between TRIM27 and MRTF-B in confluent monolayers of A431 cells.

(D) Effect of depletion or inhibition of E-cadherin on RhoA activity. A431 cells were transfected or treated with indicated reagents and seeded for confluence in a monolayer. siEcad, small interfering E-cadherin.

(E) Effects of Rho suppression on integrin β1 upregulation in LCs. A431 cells were treated with the Rho inhibitor C3T or vehicle as a control. Two hours after treatment, cell monolayers were scratched and incubated with the reagents for the indicated time.

(F) Effects of Rho suppression on the interaction between TRIM27 and MRTF-B. Monolayers of A431 cells treated with C3T or untreated A431 cells were scratched and incubated for 6 hr, followed by immunoprecipitation with anti-MRTF-B.

(G) Effects of RhoE and DDR1 on TRIM27/MRTF-B-dependent upregulation of integrin β1 in the confluent monolayer. A431 cells were transfected with the indicated combination of siRNAs and reagents and seeded to form a confluent monolayer, followed by western blot analysis. Error bars represent the mean ± SD from three independent experiments. *$p < 0.05$. siDDR1, small interfering DDR1; siC3T, small interfering C3T; siRhoE, small interfering RhoE.

(H) A schematic illustration of the model proposed in this study. For details, see the text of the article.

led to an increase in integrin β1 expression, regardless of the density of A431 cells, which was dependent on the expression of MRTF-B and TRIM27 (Figures 3A and 3B). Additionally, E-cadherin depletion by RNAi increased MRTF-B/TRIM27 interactions in confluent A431 cells (Figure 3C). Taken together, these data suggested that the loss of cadherin-mediated intercellular adhesion, found at the leading edge of LCs in cancer cell groups, led to the formation of the MRTF-B/TRIM27 complex in order to regulate integrin β1 expression.

The activation of RhoA is regulated by E-cadherin-mediated cell-cell adhesion (Figure 3D; Hidalgo-Carcedo et al., 2011; Busche et al., 2008; Charbonney et al., 2011; Fan et al., 2007). Interestingly, we found that integrin β1 upregulation, the nuclear translocation of MRTF-B, and MRTF-B/TRIM27 interactions were impaired by treatment with a Rho inhibitor, C3 transferase (C3T) (Figures 3E, 3F, and S3B). A previous study showed that the regulation of RhoA activity by E-cadherin requires the localization of RhoE, an endogenous RhoA inhibitor, at the point of cell-cell contact (Hidalgo-Carcedo et al., 2011). Consistent with this notion, depletion or inhibition of E-cadherin resulted in cytoplasmic translocation of RhoE (Figure S3C). In addition, depletion of RhoE or discoidin domain receptor 1 (DDR1), which is also involved in E-cadherin-mediated inhibition of RhoA at the point of cell-cell contact (Hidalgo-Carcedo et al., 2011), caused an increase in integrin β1 expression in confluent A431 cells, which was reversed by TRIM27 or MRTF-B depletion (Figure 3G). Because RhoA functions to change the dynamic equilibrium of

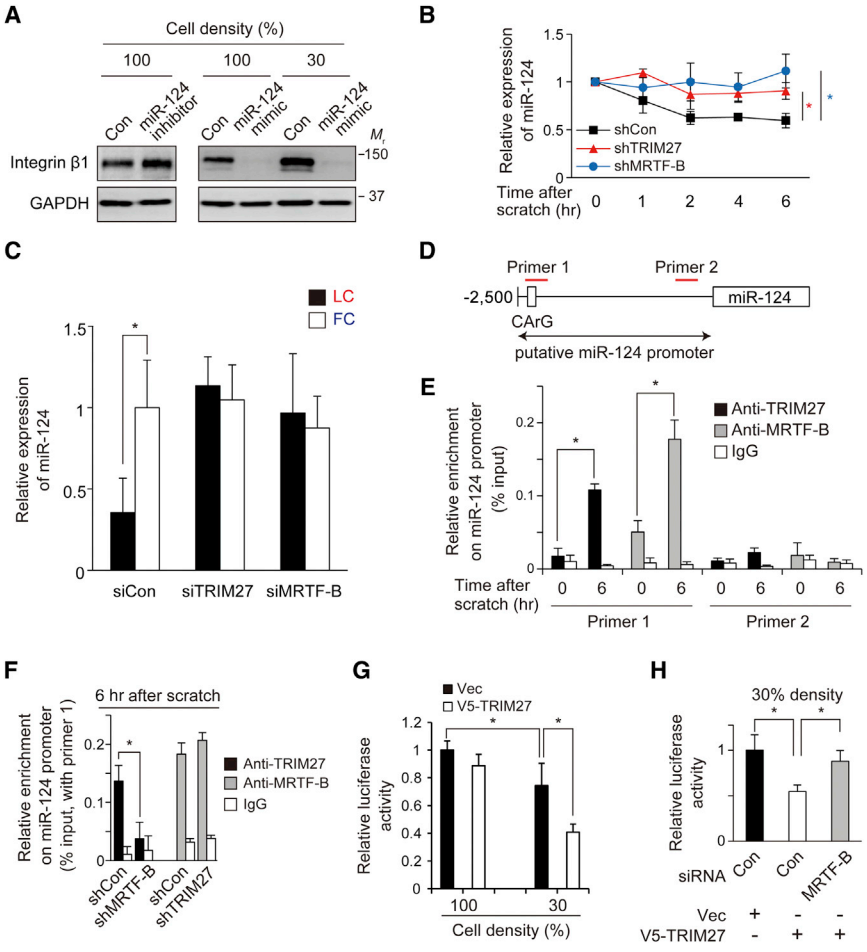

Figure 4. Suppression of *miR-124* by TRIM27/MRTF-B Mediated LC-Specific Integrin β1 Expression

(A) *miR-124* targeted *ITGB1* mRNA. A431 cells were transfected with control (Con), *miR-124* inhibitor, or mimic and seeded at the indicated cell density.

(B) Effects of TRIM27 or MRTF-B depletion on *miR-124* expression during sheet migration. Quantification of the amount of *miR-124* in cells at the indicated time after scratching was carried out using qPCR. The data are the mean ± SD from three independent experiments.

(C) Effects of TRIM27 or MRTF-B depletion on *miR-124* expression in LCs and FCs. RNA was isolated from LCs and FCs among collectively migrating A431 cells transfected with the indicated siRNA and analyzed using qPCR.

(D) Schematic illustration of the putative *miR-124* promoter region. Red bars indicate the location of the primers used for ChIP. CArG, consensus binding site for SRF.

(E) Time-dependent recruitment of TRIM27 and MRTF-B to the *miR-124* promoter in LCs during sheet migration. ChIP-qPCR was performed with anti-TRIM27 or anti-MRTF-B antibodies.

(F) Recruitment of TRIM27 to the *miR-124* promoter in LCs by MRTF-B. The indicated cells were analyzed using ChIP-qPCR with the indicated antibodies at 6 hr after scratching the cell monolayers.

(G and H) Effects of TRIM27 on *miR-124* promoter activity in cells seeded to form nests, dependent on MRTF-B. Luciferase reporter assays were performed with a reporter vector containing the *miR-124* promoter.

All error bars in this figure represent mean ± SD. *$p < 0.05$.

G-actin and F-actin, which subsequently increases the level of G-actin-free MRTF proteins to promote its nuclear translocation (Posern and Treisman, 2006; Olson and Nordheim, 2010), our data implied that changes in RhoA activity following the loss of intercellular adhesion mediated MRTF-B/TRIM27-dependent integrin β1 expression in LCs (Figure 3H).

Suppression of *miR-124* by TRIM27/MRTF-B Mediated LC-Specific Integrin β1 Expression

Next, we addressed the mechanisms acting downstream of the MRTF-B/TRIM27 complex. Chromatin IP (ChIP) conducted to measure histone H3 acetylation, a marker for active promoters, at the integrin β1 gene (*ITGB1*) locus revealed no apparent changes in *ITGB1* promoter activity in collectively migrating A431 cells after scratching, suggesting that integrin β1 expression was not transcriptionally regulated (Figure S4A). Fractionation of mRNA into translatable polysome and non-polysome fractions revealed that integrin β1 translation was activated during collective sheet migration in A431 cells (Figure S4B). Furthermore, the half-life of *integrin β1* mRNA in A431 cells seeded at a low density was shortened by MRTF-B or TRIM27 depletion (Figure S4C). These results suggested that the MRTF-B/TRIM27 complex regulated integrin β1 expression by altering its mRNA stability and/or the rate of translation.

Given the effects of microRNAs (miRs) on the regulation of mRNA stability and translation (Filipowicz et al., 2008), we investigated the involvement of *miR-124*, an miR reported to target the 3′ UTR of integrin β1 mRNA (Cao et al., 2007; Hunt et al., 2011), in mediating integrin β1 expression in LCs. *miR-124* suppressed integrin β1 expression, as shown in A431 cells transfected with an *miR-124* inhibitor or its mimic (Figure 4A). Quantitative PCR (qPCR) for *miR-124* showed that *miR-124* transcription was decreased in a time-dependent manner during sheet migration in control cells, but not in MRTF-B- or TRIM27-depleted cells, suggesting that the MRTF-B/TRIM27 complex suppressed *miR-124* expression (Figure 4B). Isolation of LCs and FCs by LCM further showed that *miR-124* was specifically downregulated in LCs, dependent on TRIM27 and MRTF-B (Figure 4C).

The regulation of *miR-124* promoter activity by TRIM27 and MRTF-B was further examined using a ChIP assay, which showed that TRIM27 and MRTF-B were associated with a putative SRF-binding sequence in the *miR-124* promoter (CArG box) (Posern and Treisman, 2006; Olson and Nordheim, 2010), not a region juxtaposed to the transcription start site, during sheet migration (Figures 4D and 4E). MRTF-B depletion

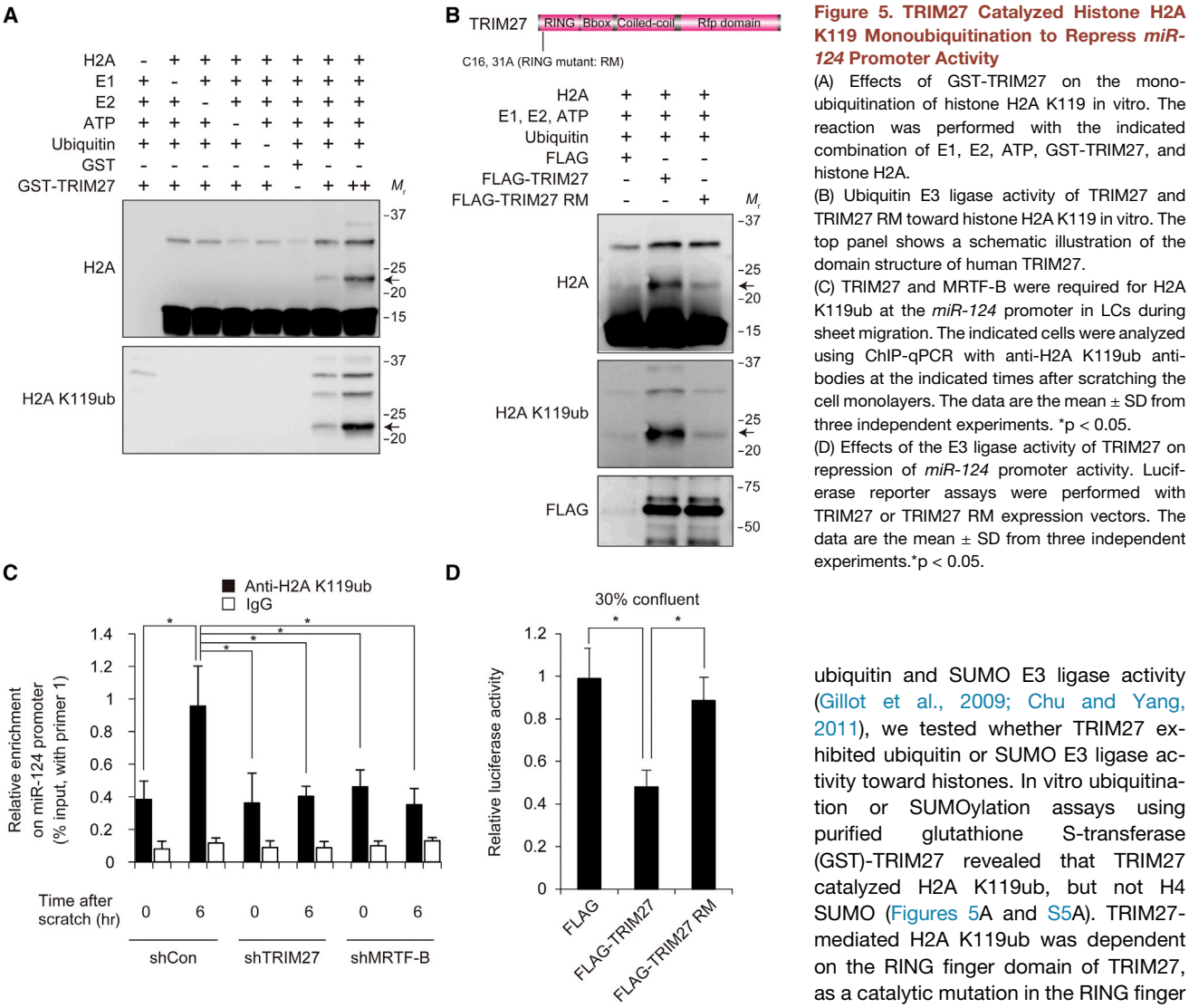

Figure 5. TRIM27 Catalyzed Histone H2A K119 Monoubiquitination to Repress *miR-124* Promoter Activity

(A) Effects of GST-TRIM27 on the mono-ubiquitination of histone H2A K119 in vitro. The reaction was performed with the indicated combination of E1, E2, ATP, GST-TRIM27, and histone H2A.

(B) Ubiquitin E3 ligase activity of TRIM27 and TRIM27 RM toward histone H2A K119 in vitro. The top panel shows a schematic illustration of the domain structure of human TRIM27.

(C) TRIM27 and MRTF-B were required for H2A K119ub at the *miR-124* promoter in LCs during sheet migration. The indicated cells were analyzed using ChIP-qPCR with anti-H2A K119ub antibodies at the indicated times after scratching the cell monolayers. The data are the mean ± SD from three independent experiments. *$p < 0.05$.

(D) Effects of the E3 ligase activity of TRIM27 on repression of *miR-124* promoter activity. Luciferase reporter assays were performed with TRIM27 or TRIM27 RM expression vectors. The data are the mean ± SD from three independent experiments.*$p < 0.05$.

markedly attenuated TRIM27 recruitment to the *miR-124* promoter, whereas MRTF-B associated with the promoter independent of TRIM27, indicating that MRTF-B recruited the repressive activity of TRIM27 to the *miR-124* promoter (Figure 4F). Supporting this view, luciferase assays using a putative *miR-124* promoter-containing construct showed that TRIM27 overexpression repressed *miR-124* promoter activity in cell nests, but not confluent cells, depending on the presence of MRTF-B (Figures 4G and 4H).

TRIM27 Repressed *miR-124* Promoter Activity through the Monoubiquitination of Histone H2A K119

We next sought to examine the mechanism through which TRIM27 repressed *miR-124* transcription. Because transcriptional repression can be mediated by monoubiquitination of histone H2A lysine 119 (H2A K119ub) and SUMOylation of histone H4 (H4 SUMO) (Shiio and Eisenman, 2003; Zhou et al., 2009; Chi et al., 2010) and TRIM27 possesses both ubiquitin and SUMO E3 ligase activity (Gillot et al., 2009; Chu and Yang, 2011), we tested whether TRIM27 exhibited ubiquitin or SUMO E3 ligase activity toward histones. In vitro ubiquitination or SUMOylation assays using purified glutathione S-transferase (GST)-TRIM27 revealed that TRIM27 catalyzed H2A K119ub, but not H4 SUMO (Figures 5A and S5A). TRIM27-mediated H2A K119ub was dependent on the RING finger domain of TRIM27, as a catalytic mutation in the RING finger (RM) led to a significant decrease in its ubiquitin ligase activity (Figure 5B). Moreover, ChIP analysis showed that depletion of TRIM27 or MRTF-B resulted in a decrease in H2A K119ub levels during sheet migration (Figure 5C), further supporting that *miR-124* promoter activity was repressed by TRIM27, but not its RM mutant, in A431 cells seeded to form nests (Figure 5D). These findings indicated that TRIM27 repressed *miR-124* promoter activity through the monoubiquitination of H2A K119.

MRTF-B and TRIM27 Enhanced the Collective Invasion and Metastasis of Cancer Cells in Both In Vitro and In Vivo Experimental Models

We next examined whether MRTF-B or TRIM27 enhanced collective movement of cancer cells. In wound-healing assays using the confluent cell monolayer and an organotypic culture model in which cancer cells were plated on top of gels containing extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs) (Gaggioli et al., 2007), MRTF-B or TRIM27 deletion

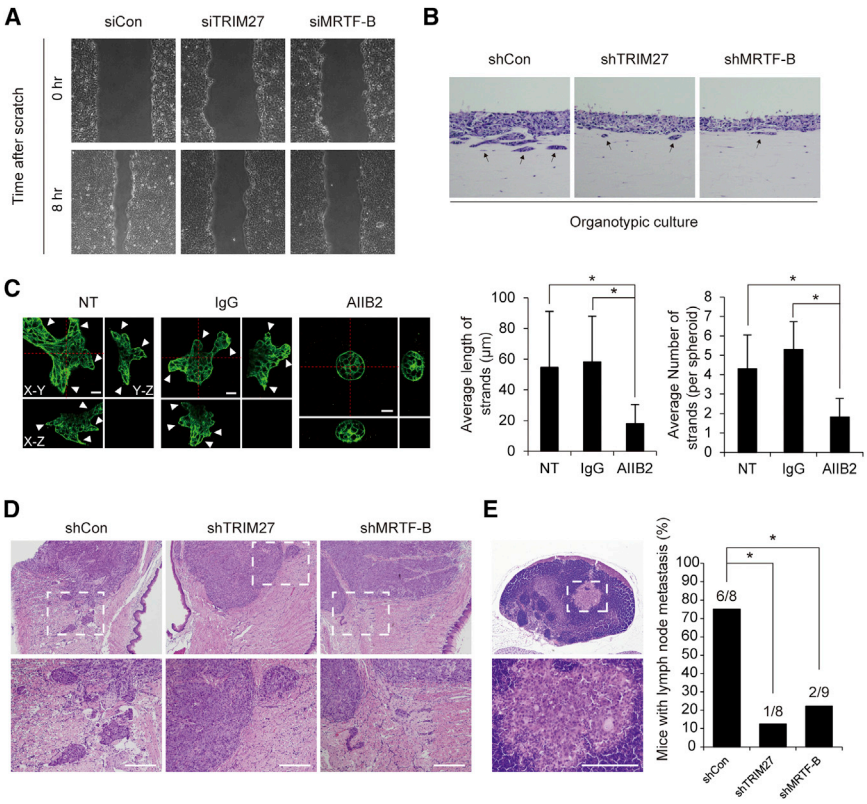

Figure 6. MRTF-B/TRIM27 Contributed to Collective Invasion and Metastasis through the Regulation of Integrin β1Expression in LCs

(A) Effects of knockdown of TRIM27 and MRTF-B on cancer cell migration. A monolayer of A431 cells transfected with the indicated siRNA was scratched and incubated. Pictures were taken at the indicated times.

(B) Effects of TRIM27 or MRTF-B depletion on collective invasion of A431 cells in an organotypic culture model. Indicated cells were placed on Matrigel/collagen mixed gels containing CAFs and incubated for 10 days.

(C) Immunostaining of A431 spheroid cultured in collagen gel. Cells incubated with the indicated antibodies (AIIB2 or control IgG) or without treatment (nontreated [NT]) were stained with Alexa 488-conjugated phalloidin (green). Cross-sectional views of the X-Y, X-Z, and Y-Z directions (axes) are shown together. Arrowheads indicate the representative strands. The scale bars represent 20 μm. The graphs show the average length (left) and number (right) of invading strands. For each group, 17 colonies from two independent experiments were measured.

(D and E) Effects of TRIM27 or MRTF-B depletion on lymph node metastasis in a mouse tongue cancer model. FaDu cells (2×10^6) expressing the indicated shRNAs were injected into mouse tongues. (D) Representative images of tissue sections stained with H&E. The regions within the dashed boxes in top panels are shown at a higher magnification in lower panels. The scale bars represent 200 μm. (E) Left panel: representative images of metastases in lymph nodes. The regions within the dashed boxes in top panels are shown at a higher magnification in lower panels. Right panel: the graph shows the rate of metastasis for each cell line. shCon, n = 8; shTRIM27, n = 8; shMRTF-B, n = 9.

All error bars represent the mean ± SD. *p < 0.05.

abrogated collective cell migration and decreased the budding of cancer cell nests (Figures 6A and 6B).

We further evaluated the mechanism of how the increased expression of integrin β1 contributes to the behavior of LCs. Given that integrin β1 is required for front-rear polarity and protrusive activity of migrating cells (Ridley et al., 2003), we assessed the polarity and lamellipodia formation of LCs by immunostaining with GM130, a marker for the Golgi apparatus, or by transmitted light microscopy. Depletion of TRIM27, MRTF-B, or integrin β1 significantly reduced the amount of polarized cells and protrusive cells (Figures S6A and S6B). To further investigate the effect of integrin β1 on the behavior of cancer cell collectives in a 3D environment, we performed a spheroid invasion assay in a collagen gel (Li et al., 2013) to assess the formation of invading strands. Inhibition of integrin β1 by the integrin β1-neutralizing antibody AIIB2, which is expected to inhibit integrin β1 at the outer rim of the spheroid, resulted in a reduction in both the length and number of the multicellular strands (Figure 6C).

Finally, we tested the importance of MRTF-B or TRIM27 on collective invasion and metastatic ability of cancer cells in a mouse tumor model. FaDu cells, a head and neck SCC cell line, were transplanted into the tongues of athymic nude mice (Figures 6D and 6E). The roles of MRTF-B and TRIM27 in the regulation of integrin β1 expression in LCs and collective migration/invasion were proved also in FaDu cells (Figures S6C–S6H). At 14 days after the transplantation, control cells exhibited collective invasion into the surrounding stroma of the tongue and spreading/budding of cancer cell nests, which were less frequent in MRTF-B- or TRIM27-depleted cells (Figure 6D). Integrin β1 levels were also higher in LCs than FCs in cancer cell nests, and this effect was abrogated by MRTF-B or TRIM27 depletion (Figure S6I). Additionally, the frequency of cervical lymph node metastasis significantly decreased with depletion of MRTF-B or TRIM27 (Figure 6E). Taken together, these findings suggested that the MRTF-B/TRIM27 complex regulated collective invasion and metastasis at least partially through the upregulation of integrin β1 in LCs.

DISCUSSION

In this study, we demonstrated the role of the MRTF-B/TRIM27 complex in the regulation of integrin β1 expression via Rho and *miR-124* in LCs among migrating cancer cell groups following loss of intracellular adhesion (Figure 7). Our findings provide a model for the generation of LCs and FCs that form heterogeneous cancer cell groups to undergo collective invasion, which may serve to enhance their invasion into surrounding stroma and metastasis to distant organs.

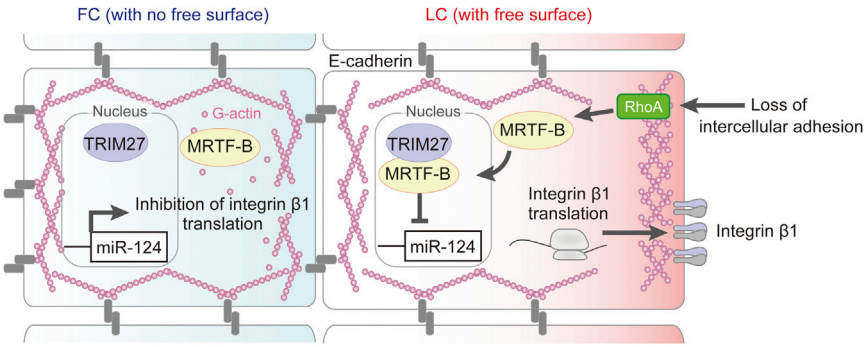

Figure 7. Proposed Model for the Differential Expression of Integrin β1 and the Generation of Heterogeneity between LCs and FCs

The MRTF-B/TRIM27 complex transmits a signal from the loss of intercellular adhesion or the presence of a free surface, thereby regulating integrin β1 expression via Rho and *miR-124* in LCs.

Although TRIM27 is overexpressed in many types of cancers (Kato et al., 2009; Tsukamoto et al., 2009; Iwakoshi et al., 2012), the expression signature and functions of MRTF proteins remain elusive. MRTF-A directly regulates integrin β1 promoter activity via its interacting proteins (Brandt et al., 2009), whereas we found that MRTF-B upregulated integrin β1 expression through the repression of *miR-124*. These data suggested the existence of the functional differences between MRTF-A and -B despite that they share interactions with SRF and some target genes (Posern and Treisman, 2006; Olson and Nordheim, 2010). Given our observation that TRIM27 specifically bound to MRTF-B, but not MRTF-A (data not shown), it will be interesting to examine the expression pattern of each MRTF protein in human cancers and correlate these results with invasiveness and malignancy.

We also found that the E3 ubiquitin ligase activity of TRIM27 was involved in determining integrin β1 expression in LCs, although the specific role of H2A K119ub in collective cancer cell invasion remains unknown. Previous studies have shown that several ubiquitin E3 ligases, including Ring1B, RNA-binding RING-dependent ubiquitin protein ligase (hRUL138), ubiquitin protein ligase E3 component n-recognin 2 (UBR2), and breast cancer 1, early onset (BRCA1), catalyze H2A K119ub for the silencing of their target genes (Braun and Madhani, 2012). We found that TRIM27 depletion had no obvious effects on global monoubiquitinated H2A K119 in cells (Figure S5B), which was in contrast to dramatic effects of Ring1B depletion on global ubiquitination of H2A K119 in cells (Wang et al., 2004). This suggested that the ligase activity of TRIM27 was spatially and temporally regulated to modulate histone modification such that the expression levels of *miR-124* and its other target genes were differentially regulated in LCs. As indicated in our study, the presence of free surfaces in cells seemed to be a cellular cue for spatial activation of TRIM27 function.

A long-standing view is that LCs have been recognized as a major source of traction force that pulls and advances the cell sheet (Vaughan and Trinkaus, 1966; Friedl et al., 2004; Gov, 2007). Although the overall contribution of LCs to the global force seems to be relatively smaller than generally expected because the traction force is generated not only in LCs but also in FCs, it is certain that LCs indeed generate higher traction force (~3-fold) than FCs, which is sufficient to locally guide FCs (Farooqui and Fenteany, 2005; Trepat et al., 2009; Tambe et al., 2011). Another recent report revealed that RhoA has a dominant role in force generation in LCs through the regulation of actomyosin contractility (Reffay et al., 2014). Considering that integrin β1 mediates the generation of traction force by interacting with ECM and transducing intracellular signaling (Ridley et al., 2003), our findings of RhoA-dependent higher integrin β1 expression in LCs provide another link between RhoA activation and the traction force generation.

The present study also demonstrated the importance of intratumor heterogeneity for cancer cells, which contributes to cancer progression (Niepel et al., 2009; Marusyk et al., 2012; Swanton, 2012). Among several mechanisms thought to generate heterogeneity in cancer cell populations, phenotypic cell-to-cell variability depends on stochastic mechanisms, including interconvertible activation of the Rho family of small GTPases, metastable configurations of intracellular networks, and chromatin alterations (Kreso et al., 2013; Sanz-Moreno et al., 2008; Spencer et al., 2009; Sharma et al., 2010; Roesch et al., 2010). Our present study suggested another nongenetic mechanism promoting cell-to-cell variability, wherein differential integrin β1 expression between LCs and FCs was regulated by protein-protein interactions that depend on the positions of individual cells in cancer cell groups. Further studies are needed to address how the heterogeneity between LCs and FCs, which does not involve genetic alterations or cell differentiation hierarchies, contributes to cancer progression and the prognosis of patients.

In studying collective invasion of cancer cells, the involvement of the cancer microenvironment should be considered (Gaggioli et al., 2007; Friedl and Alexander, 2011; Friedl et al., 2012). Therefore, future challenges include understanding how intrinsic machineries, such as the mechanism shown in this study, cooperate with other determinants including extrinsic stimuli derived from the cancer stroma for the production of distinct cell states (Bhowmick et al., 2004). Collective cell migration is also a general feature of morphogenesis during embryonic development (Friedl and Gilmour, 2009; Gray et al., 2010; Friedl et al., 2012; Rørth, 2012). A recent study revealed that nascent neural crest cells, which are also composed of LCs and FCs, migrate collectively during embryogenesis and that a gradient of extrinsic growth factors contributes to the generation of the multicellular polarity and heterogeneity of the cell groups (McLennan et al., 2012). Considering that cancer cells recapitulate the behavior of cells during embryonic morphogenesis in a dysregulated manner, it is important to elucidate the differences in the mechanisms of collective cell migration between cancer cells and nontransformed cells during development.

EXPERIMENTAL PROCEDURES

LCM

Cells seeded to form nests on type I collagen-coated foil-bottom dishes (WillCo Wells) were fixed with methanol (for protein extraction) or acetone (for RNA extraction). Microdissection was performed using an LMD7000 (Leica Microsystems) system. LCs were isolated by dissecting cells in the first row. Cells more than five cells behind the LCs were collected as FCs. Dissected cells were collected into 200 μl tubes. Then, the isolated cells were subjected to protein extraction using Laemmli SDS sample buffer or RNA extraction for qPCR using an RNeasy mini kit (Life Technologies).

Protein Purification and In Vitro Ubiquitination Assay

TRIM27 cDNA was subcloned into pGEX KG vector and expressed in *E. coli*. Bacterial cells were then resuspended and sonicated in TDZ buffer (20 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol [DTT], 50 μM $ZnCl_2$) on ice. GST-tagged TRIM27 and GST protein were purified by glutathione-sepharose 4B beads (GE Healthcare).

For in vitro ubiquitination of histone H2A, 100 ng of recombinant GST or GST-TRIM27 protein was mixed with 500 ng purified histone H2A, 150 nM UBE1 (E1 enzyme), 500 nM UbcH5a (E2 enzyme), 5 mM ATP, and 150 μM ubiquitin. The reactions were incubated at 37°C for 2 hr in 50 mM Tris-HCl (pH 7.5), 50 μM $ZnCl_2$, 5 mM $MgCl_2$, and 1 mM DTT. Following boiling in SDS sample buffer, proteins were resolved by SDS-PAGE (13%) and subjected to western blot analysis with anti-histone H2A or anti-monoubiquitinated H2A K119.

Human embryonic kidney 293 (HEK293) cells were cotransfected with plasmids encoding FLAG-tagged TRIM27, TRIM27 RM, or empty vector and harvested 48 hr after transfection. Cells were then lysed in radioimmunoprecipitation assay buffer and incubated with anti-FLAG M2 agarose affinity gel (Sigma-Aldrich). Purified proteins were used for in vitro ubiquitination assays as described above.

Organotypic Culture

The organotypic assay was carried out as described elsewhere (Hidalgo-Carcedo et al., 2011; Gaggioli et al., 2007). Lung-cancer-associated fibroblasts (5×10^5; Cellular Engineering Technologies) were embedded in a mixture of type I collagen (BD Biosciences) and Matrigel (BD Biosciences), yielding a final collagen concentration of 4.6 mg/ml and a final Matrigel concentration of 2.2 mg/ml. The gel was incubated at 37°C for 2 hr in 24-well plates, and 5×10^5 A431 cells were plated on top in serum-free medium for 24 hr. Gels were then mounted on 6-well chambers and fed from underneath with complete medium. After 10 days, the cultures were fixed with 4% paraformaldehyde plus 0.25% glutaraldehyde in PBS and stained according to standard methods for hematoxylin and eosin (H&E) staining.

Spheroid Invasion Assay of Squamous Cell Carcinoma

A glass dish with a radius of 8 mm was coated with 50 μl of collagen solution (1.9 mg/ml) and incubated for 20 min at 37°C to complete gelation. Then, 10 μl of 1.0×10^4 cells in suspension was mixed thoroughly with 200 μl of collagen solution and plated on the lower layer of the collagen gel. After collagen gelation at 37°C for 20 min, the cell-collagen mixture was covered with 250 μl of fetal-bovine-serum-containing medium and cultured for 3 days to form spheroids. Then, control immunoglobulin G (IgG; Sigma-Aldrich) or AIIB2 (1 μg/ml) was added to the culture medium. The antibody-containing medium was changed every 2 days. After 11 days of culturing, the cells were fixed by paraformaldehyde, permeabilized by 0.5% Triton X-100, and stained with Alexa Fluor 488 phalloidin. Phalloidin-labeled spheroids were imaged with confocal microscopic collection of a stack of images along the z axis with a 1 μm interval between optical sections. Protrusions between two inflection points with a length of more than 10 μm were considered to be invading strands. Image processing and analysis were performed using ImageJ (National Institutes of Health) or NIS Element (Nikon) software.

Nude Mouse Tongue Cancer Model

Female athymic nude BALB/c mice, 8–10 weeks of age, were purchased from the Chubu Kagaku Shizai and housed in a specific pathogen-free animal facility. The animals were fed irradiated mouse chow and autoclaved reverse osmosis-treated water. All of the animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Usage Committee of Nagoya University. Before injection, mice were anesthetized with sodium pentobarbital (50 mg/kg body weight). Cells (2×10^6) were prepared in 20 μl serum-free Dulbecco's modified Eagle's medium and injected into the tongues of experimental animals. Two weeks after injection, tongues and cervical lymph nodes were removed from sacrificed mice, fixed in formalin, embedded in paraffin, and sectioned. Sections were then stained with H&E or anti-integrin β1 antibodies. The number of mice with lymph node metastasis were counted and statistically analyzed.

Statistical Analysis

Data are based on at least three independent experiments and are presented as the mean ± SD. Student's t tests were used for comparisons between two groups. One-way ANOVA with post hoc Tukey-Kramer tests were used for comparisons of more than three groups. Fisher's exact test was used to compare the rate of MRTF-B nuclear localization and lymph node metastasis. Differences with p values of less than 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.068.

AUTHOR CONTRIBUTIONS

T.K., A.E., and M.T. designed experiments, analyzed data, and wrote the manuscript. T.K. implemented most of the experiments. T.W., Y.K., K.F., T.U., S.M., L.W., M.I.-T., M.A., N.A., K.K., and Y.M. assisted biochemical and cell biological experiments. H.H. and S.I. performed the 3D collagen matrix invasion assay.

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